PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 11 June 1999 (11.06.99)	
International application No. PCT/EP98/06286	**Applicant's or agent's file reference**
International filing date (day/month/year) 02 October 1998 (02.10.98)	**Priority date** (day/month/year) 08 October 1997 (08.10.97)
Applicant GUILLOT, Emmanuelle et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

29 March 1999 (29.03.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

A. Karkachi

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/EP 98/ 06286	International filing date *(day/month/year)* 02/10/1998	(Earliest) Priority Date *(day/month/year)* 08/10/1997
Applicant SUEZ LYONNAISE DES EAUX et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/06286

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WAGNER M ET AL.: "In situ identification of ammonia-oxidizing bacteria" SYSTEMATIC AND APPLIED MICROBIOLOGY, vol. 18, 1995, pages 251-264, XP002068767 see the whole document ---	1,3, 5-11, 13-23
X	DE LOS REYES ET AL.: "Group-specific small-subunit rRNA hybridization probes to characterize filamentous foaming in activated sludge systems" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 63, no. 3, 1997, pages 1107-1117, XP002068768 cited in the application see the whole document ---	1-11,13, 16-18, 21-23
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 March 1999	09/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Knehr, M

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	MANZ W ET AL: "IN SITU CHARACTERIZATION OF THE MICROBIAL CONSORTIA ACTIVE IN TWO WASTEWATER TREATMENT PLANTS" WATER RESEARCH, vol. 28, no. 8, 1 August 1994, pages 1715-1723, XP000446344 see the whole document	1,3, 5-11, 16-19, 21-23
X	WAGNER M ET AL.: "Probing activated sludge with oligonucleotides specific for proteobacteria: Inadequacy of culture-dependent methods for describing microbial community structure" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 59, no. 5, 1993, pages 1520-1525, XP002068769 see the whole document	1,3, 5-10,17, 18,21-23
X	MOBARRY B K ET AL.: "Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 62, no. 6, 1996, pages 2156-2162, XP002068770 cited in the application see abstract see page 2156, column 1, paragraph 1 - column 2, paragraph 3 see page 2159, column 2, paragraph 2 - page 2161, column 2, paragraph 3; figures 1,2; tables 1,3	1-3,5, 17-19, 21-23
X	WAGNER M ET AL.: "Development of an rRNA-targeted oligonucleotide probe specific for the genus Acinobacter and its application for in situ monitoring in activated sludge" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 60, no. 3, 1994, pages 792-800, XP002097846 see the whole document	1,5,7,8, 14,15, 17,18
X	MOBARRY B K ET AL.: "Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 62, no. 6, 1997, page 815 XP002068771 cited in the application see page 815, line 1 - line 2	1,2
X	US 5 426 025 A (REEVES ROBERT H ET AL) 20 June 1995 * spécialement colonne 5, ligne 8 à colonne 6, ligne 14 * see the whole document	1,3,5, 17,18,20

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	LEMMER (M) H ET AL: "Denitrification in a methanol-fed fixed-bed reactor. Part 2: composition and ecology of the bacterial community in the biofilms" WATER RESEARCH, vol. 31, no. 8, August 1997, page 1903-1908 XP004081404 see abstract see page 1903, column 1, paragraph 1 - page 1904, column 1, paragraph 1 ---	1,18,19, 21-23
X	WO 88 03957 A (GEN PROBE INC) 2 June 1988 see abstract; claim 219 ---	1,3-5
X	WO 96 19585 A (HEIDELBERG REPATRIATION HOSPIT ;GUERTLER VOLKER (AU)) 27 June 1996 * page 29, tableau 4 * -----	1,3-5

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 98/06286

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5426025 A	20-06-1995	US 5607835 A	04-03-1997
WO 8803957 A	02-06-1988	AT 163680 T	15-03-1998
		AU 616646 B	07-11-1991
		AU 1041988 A	16-06-1988
		CA 1339871 A	19-05-1998
		DE 3752172 D	09-04-1998
		DE 3752172 T	02-07-1998
		DK 413788 A	23-09-1988
		EP 0272009 A	22-06-1988
		ES 2112824 T	16-04-1998
		FI 883482 A	22-07-1988
		JP 10042880 A	17-02-1998
		JP 1503356 T	16-11-1989
		KR 9511719 B	09-10-1995
		PT 86204 B	07-11-1990
		US 5541308 A	30-07-1996
		US 5595874 A	21-01-1997
		US 5547842 A	20-08-1996
		US 5593841 A	14-01-1997
		US 5683876 A	04-11-1997
		US 5677127 A	14-10-1997
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		US 5677129 A	14-10-1997
		US 5827651 A	27-10-1998
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		US 5693469 A	02-12-1997
		US 5679520 A	21-10-1997
		US 5714321 A	03-02-1998
		US 5674684 A	07-10-1997
		US 5840488 A	24-11-1998
WO 9619585 A	27-06-1996	AU 1307595 A	10-07-1996

REPUBLIQUE FRANÇAISE

INSTITUT NATIONAL
de la
PROPRIETE INDUSTRIELLE

RAPPORT DE RECHERCHE PRELIMINAIRE

établi sur la base des dernières revendications déposées avant le commencement de la recherche

N° d'enregistrement national

FA 549122
FR 9712552

DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie	Citation du document avec indication, en cas de besoin, des parties pertinentes	Revendications concernées de la demande examinée
X	WAGNER M ET AL.: "In situ identification of ammonia-oxidizing bacteria" SYSTEMATIC AND APPLIED MICROBIOLOGY, vol. 18, 1995, pages 251-264, XP002068767 * le document en entier * ---	1-15,17, 19,20, 22-24
D,X	DE LOS REYES ET AL.: "Group-specific small-subunit rRNA hybridization probes to characterize filamentous foaming in activated sludge systems" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 63, no. 3, 1997, pages 1107-1117, XP002068768 * le document en entier * ---	1-9, 12-15, 17-19, 22-24
X	MANZ W ET AL: "IN SITU CHARACTERIZATION OF THE MICROBIAL CONSORTIA ACTIVE IN TWO WASTEWATER TREATMENT PLANTS" WATER RESEARCH, vol. 28, no. 8, 1 août 1994, pages 1715-1723, XP000446344 * le document en entier * ---	1-9, 12-15, 17,19, 20,22-24
X	WAGNER M ET AL.: "Probing activated sludge with oligonucleotides specific for proteobacteria: Inadequacy of culture-dependent methods for describing microbial community structure" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 59, no. 5, 1993, pages 1520-1525, XP002068769 * le document en entier * --- -/--	1-7, 13-15, 17,19, 22-24

DOMAINES TECHNIQUES RECHERCHES (Int.CL.6)

C12Q

Date d'achèvement de la recherche	Examinateur
19 juin 1998	Knehr, M

CATEGORIE DES DOCUMENTS CITES

X : particulièrement pertinent à lui seul
Y : particulièrement pertinent en combinaison avec un autre document de la même catégorie
A : pertinent à l'encontre d'au moins une revendication ou arrière-plan technologique général
O : divulgation non-écrite
P : document intercalaire

T : théorie ou principe à la base de l'invention
E : document de brevet bénéficiant d'une date antérieure à la date de dépôt et qui n'a été publié qu'à cette date de dépôt ou qu'à une date postérieure.
D : cité dans la demande
L : cité pour d'autres raisons

& : membre de la même famille, document correspondant

EPO FORM 1503 03.82 (P04C13)

REPUBLIQUE FRANÇAISE

INSTITUT NATIONAL
de la
PROPRIETE INDUSTRIELLE

RAPPORT DE RECHERCHE PRELIMINAIRE

établi sur la base des dernières revendications déposées avant le commencement de la recherche

N° d'enregistrement national

FA 549122
FR 9712552

DOCUMENTS CONSIDERES COMME PERTINENTS		Revendications concernées de la demande examinée
Catégorie	Citation du document avec indication, en cas de besoin, des parties pertinentes	
D,X	MOBARRY B K ET AL.: "Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 62, no. 6, 1996, pages 2156-2162, XP002068770 * abrégé * * page 2156, colonne 1, alinéa 1 - colonne 2, alinéa 3 * * page 2159, colonne 2, alinéa 2 - page 2161, colonne 2, alinéa 3; figures 1,2; tableaux 1,3 * ---	1,2, 13-17, 19,20, 22-24
D,X	MOBARRY B K ET AL.: "Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 62, no. 6, 1997, page 815 XP002068771 * page 815, ligne 1 - ligne 2 * ---	1,16
X	US 5 426 025 A (REEVES ROBERT H ET AL) 20 juin 1995 * spécialement colonne 5, ligne 8 à colonne 6, ligne 14 * * le document en entier * ---	1,2, 13-15, 17,19,21
X	LEMMER^(M) H ET AL: "Denitrification in a methanol-fed fixed-bed reactor. Part 2: composition and ecology of the bacterial community in the biofilms" WATER RESEARCH, vol. 31, no. 8, août 1997, page 1903-1908 XP004081404 * abrégé * * page 1903, colonne 1, alinéa 1 - page 1904, colonne 1, alinéa 1 * --- -/--	1,19,20, 22-24

DOMAINES TECHNIQUES RECHERCHES (Int.CL.6)

Date d'achèvement de la recherche	Examinateur
19 juin 1998	Knehr, M

CATEGORIE DES DOCUMENTS CITES

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Y : particulièrement pertinent en combinaison avec un autre document de la même catégorie
A : pertinent à l'encontre d'au moins une revendication ou arrière-plan technologique général
O : divulgation non-écrite
P : document intercalaire

T : théorie ou principe à la base de l'invention
E : document de brevet bénéficiant d'une date antérieure à la date de dépôt et qui n'a été publié qu'à cette date de dépôt ou qu'à une date postérieure.
D : cité dans la demande
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& : membre de la même famille, document correspondant

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RAPPORT DE RECHERCHE PRELIMINAIRE

établi sur la base des dernières revendications déposées avant le commencement de la recherche

N° d'enregistrement national

FA 549122
FR 9712552

DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie	Citation du document avec indication, en cas de besoin, des parties pertinentes	Revendications concernées de la demande examinée
X	WO 91 00926 A (MICROPROBE CORP) 24 janvier 1991 * page 25, ligne 23 * ---	1,18
X	WO 96 19585 A (HEIDELBERG REPATRIATION HOSPIT ;GUERTLER VOLKER (AU)) 27 juin 1996 * page 29, tableau 4 * -----	1,18

DOMAINES TECHNIQUES RECHERCHES (Int.CL.6)

1

Date d'achèvement de la recherche	Examinateur
19 juin 1998	Knehr, M

CATEGORIE DES DOCUMENTS CITES

X : particulièrement pertinent à lui seul
Y : particulièrement pertinent en combinaison avec un autre document de la même catégorie
A : pertinent à l'encontre d'au moins une revendication ou arrière-plan technologique général
O : divulgation non-écrite
P : document intercalaire

T : théorie ou principe à la base de l'invention
E : document de brevet bénéficiant d'une date antérieure à la date de dépôt et qui n'a été publié qu'à cette date de dépôt ou qu'à une date postérieure.
D : cité dans la demande
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.....

& : membre de la même famille, document correspondant

EPO FORM 1503 03.82 (P04C13)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
AA/CA 59.172		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP98/06286	02/10/1998	08/10/1997
International Patent Classification (IPC) or national classification and IPC		
C12Q1/68		
Applicant		
SUEZ LYONNAISE DES EAUX et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
29/03/1999	25.08.99
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. (+49-89) 2399-0 Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Bradbrook, D Telephone No. (+49-89) 2399

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP98/06286

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-14 as originally filed

Claims, No.:

1-23 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-23
	No:	Claims
Inventive step (IS)	Yes:	Claims 1-23
	No:	Claims
Industrial applicability (IA)	Yes:	Claims 1-23
	No:	Claims

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Section V

Claim 1 is directed to a method of quantitative and qualitative analysis of microbes in a sample. The method comprises:

1. contacting the microbes with at least one RNA-targeted oligonucleotide probe under conditions favourable to in situ hybridization in whole cells
2. extracting those probes which have become hybridized by separation from their target and elution outside said cells
3. detecting the extracted probes and measuring their respective amounts.

It seems that none of the documents cited in the International Search Report discloses all of the technical features of claim 1. As pointed out in the description (p.3-4), the present application provides a method which overcomes some of the problems associated with the methods of the prior art, in particular hybridization assays of extracted and immobilized nucleic acids, and fluorescent in situ hybridization. None of the prior art uses a method in which an in situ hybridized probe is separated from its target and eluted from the cells for quantisation. Moreover, no indication is given in the prior art that such an approach may be used.

Therefore, claim 1, and dependent claims 2-23, appear to be new and inventive (Article 33(2) and (3) PCT).

Section VIII

The following objections to clarity arise under Article 6 PCT:

a. Use of the term "potentially" in claims 1, 6 and 14 introduces ambiguity and should be deleted: only those microbes actually in the sample are being analysed.
b. The term "on the order of" in claim 11 is vague and should be deleted.
c. In claim 14, the phrase "denaturation of every all probe" should be read "denaturation of every probe".

It should be noted that the terms "notably" and "such as" have no limiting effect on the scope of the claims in which they are used, so that any feature following either of these expressions is regarded as entirely optional (PCT Guidelines, C-III, 4.6).

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68

A3

(11) International Publication Number: WO 99/18234

(43) International Publication Date: 15 April 1999 (15.04.99)

(21) International Application Number: PCT/EP98/06286

(22) International Filing Date: 2 October 1998 (02.10.98)

(30) Priority Data:
97/12552 8 October 1997 (08.10.97) FR

(71) Applicants *(for all designated States except US)*: SUEZ LYONNAISE DES EAUX [FR/FR]; 72, avenue de la Liberté, F–92753 Nanterre Cedex (FR). NORTHWESTERN UNIVERSITY [US/US]; 1801 Maple Avenue, Evanston, IL 60201 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: GUILLOT, Emmanuelle [FR/FR]; 13, avenue Charles de Gaulle, F–78230 Le Pecq (FR). URBAIN, Vincent [FR/FR]; 7, route de Sartrouville, F–78110 Le Vésinet (FR). MANEM, Jacques [FR/FR]; Thebout, Alles sur Dordogne, F–24480 Le Buisson de Cadouin (FR). RITTMANN, Bruce, E. [US/US]; Apartment H2, 728 Noyes Street, Evanston, IL 60201 (US). STAHL, David, A. [US/US]; 2119 Payne Street, Evanston, IL 60201 (US). FLAX, Jodi [US/US]; Apartment 9A, 3470 North Lake Shore Drive, Chicago, IL 60657 (US). WAGNER, Michaël [DE/DE]; Einsteinstrasse 34, D–81675 Münich (DE).

(74) Agents: ARMENGAUD, Alain et al.; Cabinet Armengaud Ainé, 3, avenue Bugeaud, F–75116 Paris (FR).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(88) Date of publication of the international search report:
28 September 2000 (28.09.00)

(54) Title: MEANS FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF MICROBIAL POPULATIONS POTENTIALLY PRESENT IN A SAMPLE

(57) Abstract

This invention relates to means of qualitative and quantitative analysis of microbial populations potentially present in a sample. These means notably comprise the use of at least one RNA–targeted oligonucleotide probe for *in situ* hybridization in whole cells; followed by the extraction of those probes which have become hybridized by separation from their target and elution from the microbial cells; as well as the detection and measurement of said extracted probes.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
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BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
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DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/06286

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WAGNER M ET AL.: "In situ identification of ammonia-oxidizing bacteria" SYSTEMATIC AND APPLIED MICROBIOLOGY, vol. 18, 1995, pages 251-264, XP002068767 see the whole document ---	1,3, 5-11, 13-23
X	DE LOS REYES ET AL.: "Group-specific small-subunit rRNA hybridization probes to characterize filamentous foaming in activated sludge systems" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 63, no. 3, 1997, pages 1107-1117, XP002068768 cited in the application see the whole document ---	1-11,13, 16-18, 21-23

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
24 March 1999	09/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Knehr, M

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X	MOBARRY B K ET AL.: "Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 62, no. 6, 1996, pages 2156-2162, XP002068770 cited in the application see abstract see page 2156, column 1, paragraph 1 - column 2, paragraph 3 see page 2159, column 2, paragraph 2 - page 2161, column 2, paragraph 3; figures 1,2; tables 1,3	1-3,5, 17-19, 21-23
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X	MOBARRY B K ET AL.: "Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 62, no. 6, 1997, page 815 XP002068771 cited in the application see page 815, line 1 - line 2	1,2
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	LEMMER (M) H ET AL: "Denitrification in a methanol-fed fixed-bed reactor. Part 2: composition and ecology of the bacterial community in the biofilms" WATER RESEARCH, vol. 31, no. 8, August 1997, page 1903-1908 XP004081404 see abstract see page 1903, column 1, paragraph 1 - page 1904, column 1, paragraph 1 ---	1,18,19, 21-23
X	WO 88 03957 A (GEN PROBE INC) 2 June 1988 see abstract; claim 219 ---	1,3-5
X	WO 96 19585 A (HEIDELBERG REPATRIATION HOSPIT ;GUERTLER VOLKER (AU)) 27 June 1996 * page 29, tableau 4 * -----	1,3-5

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Information on patent family members

International Application No
PCT/EP 98/06286

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68

A2

(11) International Publication Number: **WO 99/18234**

(43) International Publication Date: 15 April 1999 (15.04.99)

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(30) Priority Data:
97/12552 8 October 1997 (08.10.97) FR

(71) Applicants *(for all designated States except US)*: SUEZ LYONNAISE DES EAUX [FR/FR]; 72, avenue de la Liberté, F–92753 Nanterre Cedex (FR). NORTHWESTERN UNIVERSITY [US/US]; 1801 Maple Avenue, Evanston, IL 60201 (US).

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
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(54) Title: MEANS FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF MICROBIAL POPULATIONS POTENTIALLY PRESENT IN A SAMPLE

(57) Abstract

This invention relates to means of qualitative and quantitative analysis of microbial populations potentially present in a sample. These means notably comprise the use of at least one RNA–targeted oligonucleotide probe for *in situ* hybridization in whole cells; followed by the extraction of those probes which have become hybridized by separation from their target and elution from the microbial cells; as well as the detection and measurement of said extracted probes.

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(51) International Patent Classification 6 : C12Q 1/68

A2

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(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: GUILLOT, Emmanuelle [FR/FR]; 13, avenue Charles de Gaulle, F–78230 Le Pecq (FR). URBAIN, Vincent [FR/FR]; 7, route de Sartrouville, F–78110 Le Vésinet (FR). MANEM, Jacques [FR/FR]; Thebout, Alles sur Dordogne, F–24480 Le Buisson de Cadouin (FR). RITTMANN, Bruce, E. [US/US]; Apartment H2, 728 Noyes Street, Evanston, IL 60201 (US). STAHL, David, A. [US/US]; 2119 Payne Street, Evanston, IL 60201 (US). FLAX, Jodi [US/US]; Apartment 9A, 3470 North Lake Shore Drive, Chicago, IL 60657 (US). WAGNER, Michaël [DE/DE]; Einsteinstrasse 34, D–81675 Münich (DE).

(74) Agents: ARMENGAUD, Alain et al.; Cabinet Armengaud Ainé, 3, avenue Bugeaud, F–75116 Paris (FR).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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(54) Title: MEANS FOR QUALITATIVE AND QUANTITATIVE ANALYSIS OF MICROBIAL POPULATIONS POTENTIALLY PRESENT IN A SAMPLE

(57) Abstract

This invention relates to means of qualitative and quantitative analysis of microbial populations potentially present in a sample. These means notably comprise the use of at least one RNA–targeted oligonucleotide probe for *in situ* hybridization in whole cells; followed by the extraction of those probes which have become hybridized by separation from their target and elution from the microbial cells; as well as the detection and measurement of said extracted probes.

TITLE:

Means for qualitative and quantitative analysis of microbial populations potentially present in a sample

This invention may be generally described as a means of qualitative and quantitative analysis of microbial populations potentially present in a sample. More specifically, it relates to a means of qualitative and quantitative analysis using RNA-targeted oligonucleotide probes.

The analysis of microbial populations potentially present is required for many types of solid and fluid samples. Some notable examples are those samples obtained from a natural or biological environment such as natural water or hot springs; samples taken from humans or animals such as blood, urine, vaginal and intestinal flora; and samples from urban, agricultural and industrial environments such as food products, industrial water, industrial effluents, municipal wastewater, industrial sludge, fermentation media, aerosols, filters or air from air conditioning systems.

Various laboratory techniques have been developed for the qualitative and quantitative analysis of microbial populations potentially present in a given sample.

One familiar technique involves a count of the microorganisms that develop after the sample (or an extract thereof) is cultured on various selective nutrient media under standard conditions. This technique is simple but entails significant risks of errors and artifacts (low specificity of morphological criteria, inability to detect viable but non-culturable microorganisms, inability to detect slow-growing microorganisms, need to maintain viability of bacteria between collection and enumeration). Moreover, this technique generally requires longer than 24 hours to yield results.

sample, or *in situ* on whole cells, generally after fixation (permeabilization) of the membrane (or wall) of the microorganisms potentially present in the sample.

However, cell lysis and the ensuing extraction and purification of the nucleic acids particularly total RNA, are delicate and time-consuming manipulations that require costly apparatus, trained personnel and strict experimental conditions, notably the prevention of contamination by nucleases during the procedure. This technique further implies the use of a solid support, such as a nylon membrane, onto which the purified nucleic acids are immobilized in such a way one can discriminate between them (*e.g.* dot-blot, slot-blot). It most generally also implies the use of radioactive probe labels, the handling of which requires special care. The cell lysis technique for RNA hybridididization is therefore ill-suited to use in routine analysis either in industry or in biological laboratories.

In situ hybridization in whole cells overcomes the need for preliminary extraction of the target nucleic acids by cellular lysis with all its associated disadvantages. The FISH (Fluorescent In Situ Hybridization) process, which employs fluorescence-labeled *r*RNA probes, is one existing *in situ* technique. This type of technique, generally involving fluorescence microscopy, provides a fast and sensitive qualitative analysis on many types of sample. Today, *r*RNA-targeted probes thus hybridized *in situ* with their target within whole cells can be quantified directly on the sample (flow cytometry, microscopy), although the method is not entirely satisfactory: quantification directly on the sample is technically costly, time-consuming, requires trained personnel and does not permit an accurate quantification of hybridized probes when the sample is complex and non-uniform (*e.g.* floc or aggregates formed by filamentous bacteria in sewage treatment sludge; samples containing naturally fluorescent microorganisms). As a result, the technique of *in situ* hybridization in whole cells using fluorescence-labeled oligonucleotide probes has, to date, remained an essentially qualitative technique that does not provide reliable quantitative results.

Ammonia-oxidizing β-Proteobacteria, the genus *Nitrobacter* or Acinetobacter or the species *Fibrobacter intestinalis,* the species *Escherichia Coli*). Probes with finer phylogenetic resolution can be derived by using the existing collections of RNA sequences. Many examples of such RNA-targeted probes are described in the prior art such as patents or patent applications, scientific publications *e.g.* Los Reyes *et al.* 1997, Appli. Environ. Microbiol. Vol. 63 n°3 p.1107-1117 ; Mobarry *et al.* 1996, Appli. Environ. Microbiol. Vol.62 n°6 p.2156-2162 ; Wagner *et al.* 1994, Appli. Environ. Microbiol. Vol. 60 n°3 p.792-800 ; Kane *et al.* Appli. Environ. Microbiol. Vol. 59 n°3 p. 682-686. Other examples of such probes can also be designed by the person skilled in the art. Advantageous probes are those which target ribosomal RNA (rRNA). Examples of such advantageous probes include Nb1000 (SEQ ID N°1) and Nso 1225 (SEQ ID N°2).

The method of the invention gives particularly accurate quantitative results when the cell numbers in said sample are equal to or greater than approximately 10^3 or 10^4 cells per ml. If desired, the microorganism concentration of a liquid sample can be increased by filtration or any other technique prior to implementing the method of the invention.

In a preferred arrangement of the invention, said microorganisms potentially present in the sample are also contacted with at least one probe, hereafter called "universal probe", serving to normalize the results obtained with probes targeting specific phylogenetic groups of microorganisms ("specific probes"). The amount of a specific probe in said sample may then be expressed as a ratio of the amount of said universal probe. Such an universal probe may thus enable the expression of *e.g.* the specific target *r*RNA as a percentage of the total *r*RNA. Examples of such "universal probes" include probes specific for any microorganism, or probes specific for bacteria, or for eukaryotes. Such "universal probes" are well-known in the art and any of them can be used as long as it enables said contacting step, and allows the desired "specific probe" normalization. Such a "universal probe" is

series of ethanol solutions of increasing concentration, for example by placing the sample in a 70%, 80% and then 95% ethanol solution.

Advantageously, said contacting phase is performed by placing the sample in contact with said at least one probe in the presence of a solution hereafter called "hybridization solution", which comprises a denaturing agent such as sodium dodecyl sulfate (SDS) at a concentration in a 0,001-0,1% range, preferably on the order of 0.01%; Tris-HCl , pH of about 8 at a concentration in a 0,001-0,1M range, preferably on the order of 0.02M; and a salt such as sodium chloride at a concentration in a 0,1-1,5M range, preferably on the order of 0.9M. Such a contacting is advantageously performed for an incubation time comprised between about 10 minutes and about 2 hours, and at an hybridization temperature, which is preferably the optimal temperature. For each oligonucleotide probe, the hybridization conditions (temperature; concentration of salts and denaturing agents) can be indeed optimized so as to improve the specificity of the oligonucleotide probe for the corresponding RNA sequences found in the target cells. When a plurality of oligonucleotide probes is used simultaneously, these hybridization conditions can be chosen so as to take into account the optimal conditions peculiar to every probe.

It is very advantageous for the extraction of said at least one probe to be performed following the removal of excess and unbound probe or of non-specifically associated probe material placed in contact, notably by washing with a solution hereafter called "wash solution". Such a "wash solution" advantageously comprises a denaturing agent such as sodium dodecyl sulfate (SDS) at a concentration in a 0,001-0,1% range, preferably on the order of 0.02%; Tris-HCl pH of about 8 at a concentration in a 0,001-0,1M range, preferably on the order of 0.02M, and a salt such as sodium chloride at a concentration in a 0,01-0,9M range, preferably on the order of 0.1M. The formulation of the « wash solution » (*e.g.* salt and denaturant nature and/or concentration) is adjusted so as to achieve the appropriate stringency ; *i.e.* the stringency necessary to the removal of non-specifically

possible to distinguish each probe used from the others during the detection step, for example by giving to each one its own specific label (*e.g.* different fluorochromes).

The method of the invention can be applied to a variety of samples. Samples for which an analysis using the method of the invention is of particular interest include those taken from fluids such as natural water, industrial water, industrial effluents, municipal wastewater, industrial sludge, thermal mud, food liquid or gel, fermentation medium, air, gas, aerosol; samples from a building ventilation duct, air conditioning duct; samples from edible solid, soil; samples from medical apparatus; human or animal samples such as blood, urine, vaginal or intestinal flora.

The method of the invention utilizes neither microbiological culture, nor microscopy, nor an *in vitro* amplification step (like PCR) and does not require any cell lysis step. It is reproducible, simple, fast (less than 3 hours), low-cost and does not require specially trained personnel. The method of the invention offers the additional advantage of being easy to automate.
The method of the invention notably provides a qualitative and quantitative measurement of the microbiological or sanitary status of said sample and, consequently, of the product from which said sample is taken. The method of the invention can therefore advantageously be combined with an alarm function relating to the quality, safety and/or sanitary monitoring of the product from which the sample is taken, notably as part of an industrial production line.

When the threshold value or set point is exceeded, the method of the invention permits the corresponding quality, safety and/or sanitary alarm to be triggered. It also permits the automatic or feedback control of a microbiological removal or enrichment process.

This invention also relates to the application of said method to *in vitro* diagnostics of infectious diseases.

The fixed sample is centrifuged after adding 1 ml of 70% ethanol over the residue and resuspending the cells. The mixture is centrifuged for 5 minutes then the supernatant is removed. This procedure is repeated with 80% ethanol and then again using 95% ethanol.

c) Hybridization step

A water bath is prepared at the hybridization temperature required by the probe being used (the temperature depends on the length and sequence of the probe). In the example reported here, the following probes were used:

Probe Nb 1000 specific to the *Nitrobacter* genus, with sequence SEQ ID n°1: 5' TGCGACCGGTCATGG 3'

Probe Nso 1225, specific to Ammonia-oxidizing β proteobacteria, with sequence SEQ ID n°2: 5' CGCCATTGTATTACGTGTGA 3'

Probe S Univ-1390, a universal probe for any microorganism, with sequence SEQ ID n°3 : 5' GACGGGCGGTGTGTACAA 3', and

Probe S Bac338, specific for bacteria, with sequence SEQ ID n°4: 5' GCTGCCTCCCGTAGGAGT 3'.

These probes were synthesized, purified by High Performance Liquid Chromatography (HPLC), then fluorescein-labeled at the 5' end. They are available from Operon Technologies of Alameda, California (USA) or, in France, from the Genset company based in Paris (among others).

The cells obtained from the dehydration step are resuspended in 400 µL of a hybridization solution comprising (for 10 mL): NaCl 5M 1.8 mL; Tris-HCl 1M 200 µL; SDS (sodium dodecyl sulfate) 5 µL; distilled excipient water 8 mL, for ten mL. After each probe is labeled by a fluorochrome, the necessary quantity of each probe is added (here, 1.5 nanomoles). The cells in the hybridization solution in contact with the probes are incubated for 10 minutes to 2 hours at the hybridization temperature. The hybridization samples are centrifuged and supernatants are removed.

CLAIMS

1. A method of qualitative and quantitative analysis of the microbial population(s) potentially present in a sample, characterized in that it comprises:

- contacting the microorganisms potentially present in said sample with at least one RNA-targeted oligonucleotide probe, hereafter called specific probe, able to target a desired microbiological population, under conditions favourable to *in situ* hybridization in whole cells,
- extracting by separation from their target and elution outside said cells those probes which have become hybridized,
- detecting the extracted probes and measuring the amount thereof or their respective amounts.

2. A method according to Claim 1, further characterized in that said at least one specific probe is chosen among the group consisting of Nb 1000 (SEQ ID N°1) and Nso 1225 (SEQ ID N°2).

3. A method according to Claim 1 or 2, further characterized in that said microorganisms potentially present in said sample are contacted with another probe, hereafter called universal probe, serving to normalize results obtained with probes targeting specific phylogenetic groups of microorganisms.

4. A method according to Claim 3, further characterized in that said universal probe is chosen among the group consisting of S Univ-1390 (SEQ ID N°3) and S Bac 338 (SEQ ID N°4).

5. A method according to any one of the preceding claims, further characterized in that said specific and/or universal probe(s) is a (are) *r*RNA-targeted probe(s).

12. A method according to any one of the preceding Claims, further characterized in that said contacting phase is performed for an incubation time of about 10 minutes to about 2 hours, and at the optimal hybridization temperature.

13. A method according to any one of the preceding claims, further characterized in that said extraction of said at least one probe is performed following removal of the excess and unbound probe or of non-specifically associated probe material placed in contact, notably by washing with a solution, hereafter called wash solution, which notably comprises a denaturing agent such as sodium dodecyl sulfate (SDS) and a salt such as sodium chloride at concentrations appropriate for achieving the stringency necessary to the removal of non-specifically associated probe.

14. A method according to any one of the preceding claims, further characterized in that said extraction is performed by placing said microorganisms potentially present under conditions enabling the denaturation of every all probe specifically associated with its target sequence, notably in the presence of an agent able to denature the probe-target duplex, and at a temperature higher than the melting temperature of the probe under consideration, notably at a temperature of approximately 100°C.

15. A method according to claim 14, further characterized in that the denaturing agent is formamide.

16. A method according to any one of the preceding claims, further characterized in that said extracted probes are concentrated, notably using

treatment of organic effluents, sewage treatment process such as treatment by activated sludge.

22. A method according to any of the preceding claims, further characterized in that it is used in the automatic or feedback control of a process relating to the removal or prevention of the development of microorganisms.

23. A method according to any of the preceding claims, characterized in that it is applied in the detection of foam formation during the implementation of activated sludge processes and/or for the feedback control of a method relating to the removal or prevention of the said foams.

(c) Number of strands: single
(d) Configuration: linear

(ii) Type of molecule: other nucleic acid
(iii) Hypothetical: yes
(iv) Antisense: no
(vii) Immediate source:
(B) Clone: Nb1225

(xi) Description of the sequence: SEQ ID n° 2:
5' CGCCATTGTA TTACGTGTGA 3'

(4) Information for SEQ ID n° 3:

(i) Characteristics of the sequence:
(a) Length: 18 base pairs
(b) Type: nucleotide
(c) Number of strands: single
(d) Configuration: linear

(ii) Type of molecule: other nucleic acid
(iii) Hypothetical: yes
(iv) Antisense: no
(vii) Immediate source:
(B) Clone: S Univ-1390

(xi) Description of the sequence: SEQ ID n° 3:
5' GACGGGCGGTGTGTACAA 3'

(5) Information for SEQ ID n° 4:

(i) Characteristics of the sequence:
(a) Length: 18 base pairs
(b) Type: nucleotide
(c) Number of strands: single
(d) Configuration: linear

(ii) Type of molecule: other nucleic acid
(iii) Hypothetical: yes
(iv) Antisense: no
(vii) Immediate source:
(B) Clone: S Bac338

(xi) Description of the sequence: SEQ ID n° 4:
5' GCTGCCTCCCGTAGGAGT 3'

PATENT COOPERATION TREATY

REC'D 30 AUG 1999
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
AA/CA 59.172		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP98/06286	02/10/1998	08/10/1997
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant SUEZ LYONNAISE DES EAUX et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
29/03/1999	25.08.99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. (+49-89) 2399-0 Tx: 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer Bradbrook, D Telephone No. (+49-89) 2399

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP98/06286

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-14 as originally filed

Claims, No.:

1-23 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-23
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-23
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-23
	No:	Claims	

2. Citations and explanations

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Section V

Claim 1 is directed to a method of quantitative and qualitative analysis of microbes in a sample. The method comprises:

1. contacting the microbes with at least one RNA-targeted oligonucleotide probe under conditions favourable to in situ hybridization in whole cells
2. extracting those probes which have become hybridized by separation from their target and elution outside said cells
3. detecting the extracted probes and measuring their respective amounts.

It seems that none of the documents cited in the International Search Report discloses all of the technical features of claim 1. As pointed out in the description (p.3-4), the present application provides a method which overcomes some of the problems associated with the methods of the prior art, in particular hybridization assays of extracted and immobilized nucleic acids, and fluorescent in situ hybridization. None of the prior art uses a method in which an in situ hybridized probe is separated from its target and eluted from the cells for quantisation. Moreover, no indication is given in the prior art that such an approach may be used.

Therefore, claim 1, and dependent claims 2-23, appear to be new and inventive (Article 33(2) and (3) PCT).

Section VIII

The following objections to clarity arise under Article 6 PCT:

a. Use of the term "potentially" in claims 1, 6 and 14 introduces ambiguity and should be deleted: only those microbes actually in the sample are being analysed.
b. The term "on the order of" in claim 11 is vague and should be deleted.
c. In claim 14, the phrase "denaturation of every all probe" should be read "denaturation of every probe".

It should be noted that the terms "notably" and "such as" have no limiting effect on the scope of the claims in which they are used, so that any feature following either of these expressions is regarded as entirely optional (PCT Guidelines, C-III, 4.6).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 98/ 06286	02/10/1998	08/10/1997
Applicant		
SUEZ LYONNAISE DES EAUX et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/06286

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WAGNER M ET AL.: "In situ identification of ammonia-oxidizing bacteria" SYSTEMATIC AND APPLIED MICROBIOLOGY, vol. 18, 1995, pages 251-264, XP002068767 see the whole document ---	1,3, 5-11, 13-23
X	DE LOS REYES ET AL.: "Group-specific small-subunit rRNA hybridization probes to characterize filamentous foaming in activated sludge systems" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 63, no. 3, 1997, pages 1107-1117, XP002068768 cited in the application see the whole document --- -/--	1-11,13, 16-18, 21-23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 March 1999	09/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Knehr, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	MANZ W ET AL: "IN SITU CHARACTERIZATION OF THE MICROBIAL CONSORTIA ACTIVE IN TWO WASTEWATER TREATMENT PLANTS" WATER RESEARCH, vol. 28, no. 8, 1 August 1994, pages 1715-1723, XP000446344 see the whole document ---	1,3, 5-11, 16-19, 21-23
X	WAGNER M ET AL.: "Probing activated sludge with oligonucleotides specific for proteobacteria: Inadequacy of culture-dependent methods for describing microbial community structure" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 59, no. 5, 1993, pages 1520-1525, XP002068769 see the whole document ---	1,3, 5-10,17, 18,21-23
X	MOBARRY B K ET AL.: "Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 62, no. 6, 1996, pages 2156-2162, XP002068770 cited in the application see abstract see page 2156, column 1, paragraph 1 - column 2, paragraph 3 see page 2159, column 2, paragraph 2 - page 2161, column 2, paragraph 3; figures 1,2; tables 1,3 ---	1-3,5, 17-19, 21-23
X	WAGNER M ET AL.: "Development of an rRNA-targeted oligonucleotide probe specific for the genus Acinobacter and its application for in situ monitoring in activated sludge" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 60, no. 3, 1994, pages 792-800, XP002097846 see the whole document ---	1,5,7,8, 14,15, 17,18
X	MOBARRY B K ET AL.: "Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 62, no. 6, 1997, page 815 XP002068771 cited in the application see page 815, line 1 - line 2 ---	1,2
X	US 5 426 025 A (REEVES ROBERT H ET AL) 20 June 1995 * spécialement colonne 5, ligne 8 à colonne 6, ligne 14 * see the whole document ---	1,3,5, 17,18,20

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	LEMMER (M) H ET AL: "Denitrification in a methanol-fed fixed-bed reactor. Part 2: composition and ecology of the bacterial community in the biofilms" WATER RESEARCH, vol. 31, no. 8, August 1997, page 1903-1908 XP004081404 see abstract see page 1903, column 1, paragraph 1 - page 1904, column 1, paragraph 1 ---	1,18,19, 21-23
X	WO 88 03957 A (GEN PROBE INC) 2 June 1988 see abstract; claim 219 ---	1,3-5
X	WO 96 19585 A (HEIDELBERG REPATRIATION HOSPIT ;GUERTLER VOLKER (AU)) 27 June 1996 * page 29, tableau 4 * -----	1,3-5

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 98/06286

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5426025 A	20-06-1995	US 5607835 A	04-03-1997
WO 8803957 A	02-06-1988	AT 163680 T	15-03-1998
		AU 616646 B	07-11-1991
		AU 1041988 A	16-06-1988
		CA 1339871 A	19-05-1998
		DE 3752172 D	09-04-1998
		DE 3752172 T	02-07-1998
		DK 413788 A	23-09-1988
		EP 0272009 A	22-06-1988
		ES 2112824 T	16-04-1998
		FI 883482 A	22-07-1988
		JP 10042880 A	17-02-1998
		JP 1503356 T	16-11-1989
		KR 9511719 B	09-10-1995
		PT 86204 B	07-11-1990
		US 5541308 A	30-07-1996
		US 5595874 A	21-01-1997
		US 5547842 A	20-08-1996
		US 5593841 A	14-01-1997
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		US 5827651 A	27-10-1998
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		US 5691149 A	25-11-1997
		US 5693469 A	02-12-1997
		US 5679520 A	21-10-1997
		US 5714321 A	03-02-1998
		US 5674684 A	07-10-1997
		US 5840488 A	24-11-1998
WO 9619585 A	27-06-1996	AU 1307595 A	10-07-1996